

EXHIBIT C

Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein

(peptide synthesis/antigenic determinant/foot-and-mouth disease virus/immune system)

H. MARIO GEYSEN*, SIMON J. BARTELING†, AND ROB H. MELOENT†

*Commonwealth Serum Laboratories, 45 Poplar Road, Parkville, Melbourne, Australia 3052; and †Central Veterinary Institute, 39 Houtribweg, Lelystad, The Netherlands

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ABSTRACT Antisera were raised against the chemically synthesized peptide corresponding to each epitope of three foot-and-mouth disease virus strains. Peptide synthesis was further used to determine which amino acid residues in each epitope are important for the specificity of antisera raised against the whole virus. The specificity of the antibody paratope for its epitope was shown to depend on structure as well as sequence. Anti-virus sera demonstrated a greater specificity for the homologous peptide than did the anti-peptide sera. Two of the three peptides were able to induce neutralizing antibodies against the homologous virus. The specificities of the antibodies present in the anti-peptide sera were also inferred from the reactions of each with related sets of peptides. The cross-reactions observed for the anti-peptide sera were readily explained in terms of the antibody specificities determined to be present. The findings also suggest that the diversity of antibodies raised against small peptides is limited and is determined by the immune system. A similar limited response to the native protein was observed, which may account for the high frequency with which anti-peptide sera react with the native homologous protein.

Antibodies to protein epitopes are usually highly specific and able to distinguish between proteins differing by only a single amino acid in the region of antibody binding (1-3). Structural changes induced by denaturation or chemical modification of a protein may result in the complete loss of binding by antisera against the native conformation (4, 5). These observations have led to the conclusion that antibody specificity is determined by both the sequence of amino acids and the conformation at the region of binding. In contrast to the relatively stable structure of a protein in solution, small peptides are thought to exist in a multiplicity of transient conformational states in dynamic equilibrium (6, 7). Antibodies raised against short peptides often react well with the native protein at the region of sequence homology, when this region is located at the surface of the molecule (8-10). It may also be necessary to restrict the conformational freedom of immunizing peptides in order to obtain antibodies of the same specificity as those induced by the proteins themselves.

To compare the specificity of the anti-protein response with the anti-peptide response, peptides were synthesized corresponding to an equivalent epitope of three foot-and-mouth disease virus (FMDV) strains, each belonging to a different serotype of the virus. It was assumed that anti-virus sera raised against different viral serotypes would not cross-react at a neutralization epitope. The diversity of antibodies directed to a single epitope was compared for each pair of

antisera (anti-peptide and anti-virus). The specificity of each of the anti-peptide sera was inferred from the relative reactivity for the homologous compared to the heterologous peptide.

A replacement set was synthesized consisting of all of the peptides derived by substituting, one at a time, all 19 alternate amino acids at each position within the parent sequence. Antibody diversity was then determined from the reaction patterns observed when each serum was allowed to react with the single residue replacement peptide set corresponding to the homologous parent peptide.

It was found that anti-peptide sera were less specific than the anti-virus sera. This observation was readily explicable in terms of the broader spectrum of antibody paratopes present in the anti-peptide sera when compared with anti-virus sera. This difference in the two types of sera was attributed to the greater conformational freedom of the peptide as compared with the region of sequence homology of a virus protein. However, it was found that the diversity of paratopes present in the anti-peptide sera was restricted, each having some common characteristics. This observation may explain the high frequency with which anti-peptide sera have been found to react with the homologous intact protein.

MATERIALS AND METHODS

Synthesis of Peptides. Peptides for the location of the epitopes on each of the immunologically important coat proteins (VP1s) of FMDV and for the determination of the specificity pattern of an antibody population were synthesized as described (11). Scanning for antibody-reactive peptides required the synthesis of every overlapping hexapeptide in the relevant protein sequence. For example, a protein of N residues translates into $(N - 5)$ overlapping hexapeptides, in which peptide no. 1 = residues 1 → 6, no. 2 = 2 → 7, ..., and no. $(N - 5) = (N - 5) → N$. Peptides were synthesized according to the amino acid sequences as translated from the respective nucleotide sequence: FMDV type O_1 (12), type A_{10} (13), and type C_1 (14).

For a replacement set of peptides, for each residue position in the parent sequence, peptides are synthesized in which all 19 alternative amino acids are substituted one at a time keeping the rest of the sequence the same. For example, a replacement set based on a parent sequence six residues long requires the synthesis of 120 peptides and includes six copies of the parent sequence as controls.

For the production of rabbit antisera, peptides corresponding to the respective epitopes (O_1 , residues 146-152; A_{10} , residues 144-150; and C_1 , residues 143-149) were synthesized by using standard solid-phase methods (15, 16). The composition of each peptide was confirmed by amino acid

analysis, and its purity was assessed by HPLC. Peptides were purified further by using ion-exchange chromatography where necessary to at least 80% purity. Peptides were synthesized with a cysteine residue added to the amino-terminal end and a lysine residue to the carboxyl-terminal end. These additions allowed the peptides to be coupled to a carrier protein from either end.

Coupling of Peptides to Carrier Proteins. Synthesized peptides were coupled by either end to one of the following carrier proteins: keyhole limpet hemocyanin, bovine serum albumin, or ovalbumin. Amino-terminal coupling was through the cysteine residue, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent (17); carboxyl-terminal coupling was through the lysine residue, using glutaraldehyde as the coupling reagent as follows: 10 mg of protein in 0.5 ml of 0.01 M phosphate buffer (pH 6.0) was preactivated with 5 μ l of glutaraldehyde for 30 min; 1 mg of peptide in 0.5 ml of phosphate buffer (pH 7.5) was added and coupling was allowed to proceed overnight.

Viruses. FMDV type O, subtype 1 (strain BFS 1860), type A, subtype 10 (strain Holland), and type C, subtype 1 (strain Detmold), were cultured in baby hamster kidney cells and purified (18).

Antisera. Antisera against intact virus particle were prepared by immunizing rabbits with 50 μ g of acetylene-amine-inactivated, density-gradient-purified virus in complete Freund's adjuvant. The animals were bled 3–4 weeks after the single inoculation. Antisera absorbed with intact virus were prepared by incubating 1500 μ g of purified whole virus with 1 ml of antiserum. After 72 hr at 4°C, virus-bound antibodies were removed by centrifugation. Rabbit antisera against protein-coupled peptides were prepared as follows: primary vaccination was with amino-terminal coupled peptide conjugates in complete Freund's adjuvant (Difco), 100 μ g of peptide per animal. Secondary vaccination was with carboxyl-terminal coupled peptide conjugates in Freund's incomplete adjuvant, 100 μ g per animal. Rabbits were injected by the intramuscular route and were bled prior to the first vaccination and again 2 weeks after the second.

Antibody-Binding Assays. (i) Peptides still coupled to the support used for their synthesis were allowed to react with antisera in an enzyme-linked immunosorbent assay (ELISA) as described (11). (ii) An ELISA test was used to detect the reaction between anti-peptide sera and whole virus (19) and between anti-peptide sera and chemically synthesized peptides. In the latter test, peptides were first coupled to a solid-phase, radiation-grafted polyethylene as used for the synthesis of peptides. Coupling was through the amino-terminal cysteine when using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide and through the carboxyl-terminal lysine when using glutaraldehyde as the coupling reagent. Bound antibody was determined with horseradish peroxidase-conjugated goat anti-rabbit IgG using *o*-phenylenediamine as the chromophore.

Virus Neutralization Test. The virus neutralization titer of the anti-peptide sera was determined in a microneutralization test (MNT) using baby hamster kidney monolayer cells (20). Briefly, 100 tissue culture 50% infective doses (TCID₅₀) of virus was added to a 1 in 2 dilution series of the anti-peptide sera and allowed to react for 1 hr at 37°C. Approximately 5000 cells were added and incubated for 3 days. Neutralization titers are given as the negative logarithm of the serum dilution corresponding to the end point of the test.

RESULTS

Comparable Epitopes Determined for Three Virus Strains. An immunologically important epitope of FMDV, type O₁, was located on coat protein VP1, in the region of amino acid residues 146–152 (11). Corresponding epitopes have been lo-

cated at residues 144–150 and 143–148 of the VP1 proteins of FMDV type A₁₀ and C₁, respectively. Fig. 1 shows the sequence and position of each epitope within a region of high amino acid variability between virus types. Antisera raised against virus gave a strong and type-specific reaction for the respective homologous peptides in an ELISA test (Table 1). Furthermore, the same sera after absorption with the homologous virus gave no detectable reaction with the homologous peptide, demonstrating that each epitope is present on the intact virus particle (data not shown).

Peptides Induce Antibodies That Recognize the Homologous Virus. Table 1 summarizes the results obtained when rabbit anti-peptide sera were tested for (i) reaction with the homologous and the heterologous peptide in an ELISA test, (ii) reaction with each strain of intact virus also in an ELISA test, and (iii) an ability to neutralize virus in a MNT. Anti-A₁₀-peptide sera were found to react in a specific manner with both the homologous peptide and virus. Anti-C₁-peptide sera were found to react with the heterologous virus type A₁₀, whereas anti-O₁-peptide sera were found to react with both virus types A₁₀ and C₁. In addition, anti-O₁- and anti-C₁-peptide sera demonstrated an even poorer ability to discriminate for the homologous peptide.

When sera raised against each of the three virus strains were allowed to react in an ELISA test with each of the peptides, type A₁₀ and type O₁ sera reacted in a specific manner with the homologous peptide, whereas type C₁ sera also reacted with the heterologous A₁₀ peptide (Table 1). Not shown are the results for the reaction between anti-virus sera and each virus type. Anti-intact-virus sera would be expected to contain antibodies directed to more than the one epitope considered in this study. ELISA results for these sera with intact virus are not comparable with the above results.

Paratope Specificity of Antibodies Present in Anti-Virus Sera. For each of the three virus types, Table 2 summarizes the ELISA results obtained for each replacement set of peptides when tested for antibody-binding activity with the homologous anti-virus serum. An amino acid of the parent sequence is considered to be a specific requirement for binding by all antibody paratopes present when replacement by any other amino acid is disallowed or limited to alternative residues similar in size (e.g., S and A) or side-chain characteristics (e.g., S and T). The composite paratope recognition pattern of a polyclonal antiserum then reflects the requirements common to all of the individual antibodies present. On this basis composite paratope recognition patterns for each of the three anti-virus sera were assigned as follows: antibodies to FMDV type O₁ as (G)-X-L-(Q)-X-L, to type A₁₀ as X-D-L-G-S-X, and to type C₁ as (D)-L-A-X-L-T. An X indicates a nonessential residue and the letters in parentheses indicate

	135		155
FMDV, type O ₁	R-Y-N-R-N-A-V-P-N-L-R-G-D-L-Q-V-L-A-Q-K-V		
FMDV, type A ₁₀	K-Y-S-T-G-G-S . . . R-S-C-D-L-G-S-I-A-A-R-V		
FMDV, type C ₁	T-Y-T-A-S-T . . . R . G-D-L-A-H-L-T-A-T-R		
Peptides synthesized	O ₁ -peptide	C-G-D-L-Q-V-L-A-K	
	A ₁₀ -peptide	C-G-D-L-G-S-I-A-K	
	C ₁ -peptide	C-D-L-A-H-L-T-A-K	

FIG. 1. The predicted amino acid sequences for position 135–155 (referred to subtype O₁) of the VP1 of the three FMDV strains, as aligned by Cheung *et al.* (14). Antigenic peptides as determined in the primary scan are underlined. Conserved residues are shown with an asterisk; deletions are indicated by a dot. Also identified are the peptides synthesized to which rabbit antisera were raised. In this paper, amino acids are identified by the single-letter code (21).

Table 1. Antigen-binding and neutralizing activity of anti-peptide and anti-whole virus sera for homologous and heterologous peptides and virus

Serum tested	Peptide*			Virus*			Virus†		
	O ₁	A ₁₀	C ₁	O ₁	A ₁₀	C ₁	O ₁	A ₁₀	C ₁
Anti-peptide serum									
Peptide O ₁	100	30	73	100	40	20	<0.6	<0.6	<0.6
Peptide A ₁₀	<10	100	10	<10	100	<10	<0.6	3.1	<0.6
Peptide C ₁	87	49	100	<10	63	100	<0.6	<0.6	2.5
Anti-virus serum									
Type O ₁	100	<10	<10				2.2	<0.6	<0.6
Type A ₁₀	<10	100	<10				<0.6	3.1	<0.6
Type C ₁	10	19	100				<0.6	<0.6	2.2

Antibody-binding activity of sera with peptides and virus is shown as the value obtained for the heterologous reaction expressed as a percentage of that for the homologous reaction. Values represent the mean of at least two sera and two determinations. A serum dilution of 1:400 was used in each case. The 100% values obtained for the anti-virus and anti-peptide sera were comparable and correspond to an absorbance in the range of 1.7–2.2. Values were corrected for background color as determined from preimmune serum controls. Neutralization titers are shown as the negative logarithm of the serum dilution corresponding to the end point of the MNT.

*In an ELISA.

†In a MNT.

that the limitation to replacement of these amino acids was dependent on the serum used in the test (data not shown).

Peptides Induce Antibodies with Limited Paratope Specificities. Rabbit anti-peptide sera were allowed to react with the peptides comprising the single amino acid replacement set for each epitope. Fig. 2 shows the ELISA activity obtained for each of the 120 peptides corresponding to the replacement set of the subtype A₁₀ peptide when allowed to react with anti-A₁₀-peptide serum. For comparison, the pattern obtained for the same peptides is shown when anti-virus, subtype A₁₀ serum was used in the test. Table 3 summarizes the results obtained for each anti-peptide serum when tested with the replacement set of peptides derived from the homologous sequence. By using the same convention as before, composite antibody paratope specificities for each anti-peptide serum were assigned as follows: to peptide O₁, as X-

(D)-L-Q-X-(L), to peptide A₁₀ as X-D-L-G-X-X, and to peptide C₁ as D-X-A-X-X-X.

INTERPRETATION OF ASSIGNED COMPOSITE PARATOPE SPECIFICITIES

It may be assumed that the results obtained in an antibody-binding assay using polyclonal sera containing a multiplicity of antibody paratopes will show the summation of the multiple interactions possible with each epitope of the protein. Competition between paratopes for a given epitope will depend on their relative concentration and on the affinity of each for that epitope. The larger the number of paratopes present, the greater the difficulty in estimating the range of individual specificities present. For example, the ELISA reaction of anti-virus type A₁₀ serum with the replacement set

Table 2. Relative antibody-binding activities of the single residue replacement sets of peptides with anti-virus serum

Substituting amino acid	Parent sequences of single residue replacement sets of peptides																	
	Subtype O ₁						Subtype A ₁₀						Subtype C ₁					
	G	D	L	Q	V	L	G	D	L	G	S	I	D	L	A	H	L	T
A	11	22			62		66			29	12	47			108	272		
C		12					66					96				68		
D		136		117	40		49	118				28	54			367		
E		92		52	63		61					81				307		
F		137			42		63					87				159		
G	88	52					105			98	27							
H	10	62					107									95		
I		21			56		60					85	131					
K	32	87		68	82		87					108				253		
L		81	88		68	105	50	28	88			108		133		50	93	
M	18	37		53	88		67					97				104		
N	24	89		49	34		69									99		
P	25	49					15											
Q	26	80		102	106		65					108				224		
R		29		10	33		57					162				101		
S	77	63		45	91		63				106	97				238		30
T	14	104			98		60				97	72				262		117
V		60			81		27					55	135			94		
W							101					23						
Y		21			14		59					19				77		

Antibody-binding activities are expressed as a percentage of the mean activity of the six parent sequences synthesized for each group. Activities are shown for all peptides for which the activity was >10%. Shown are the values obtained for anti-intact-virus serum when allowed to react with the homologous set of peptides. Activities shown in boldface type correspond to the values for the parent sequence. One-hundred percent values for anti-virus sera types A₁₀, O₁, and C₁ correspond to absorbances of 1.75, 0.93, and 0.62, respectively.

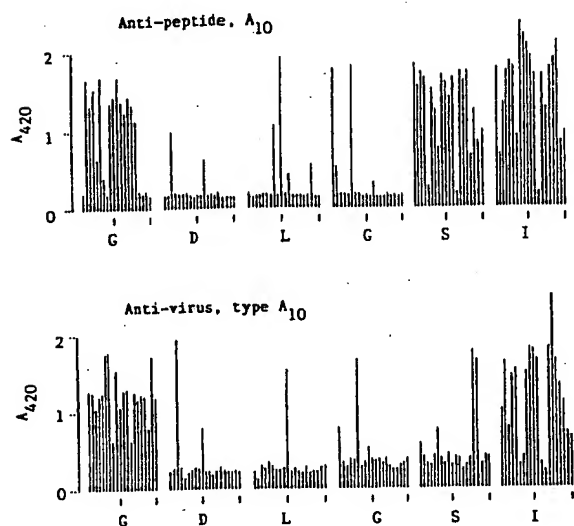


FIG. 2. The antibody-binding activity for each peptide is shown as a vertical line proportional to the ELISA extinction obtained. Every group of 20 lines corresponds to the complete replacement set for one of the six amino acid positions in the hexapeptide G-D-L-G-S-I. Within each group of 20 lines the left-hand line corresponds to the substitution of the original residue by alanine, and the successive lines are then in alphabetic order according to the single-letter code for each amino acid. The upper group shows the results obtained when a rabbit antiserum raised against the intact virus was used in the test; the lower group shows results obtained when the antiserum had been raised against the homologous peptide.

of peptides based on the A_{10} -peptide could be the summation of the following individual paratope specificities: D-L-G-S, G-D-L-G-S, D-L-G-S-I, and G-D-L-G-S-I. In contrast, the response of anti-peptide type A_{10} serum could be the summation of the following individual paratope specificities: D-L-G (assuming three residues are sufficient for interaction), G-D-L-G, D-L-G-S, D-L-G-X-I (all four residue specific-

ities), G-D-L-G-S, G-D-L-G-X-I, D-L-G-S-I (all five residue specificities), and, last, G-D-L-G-S-I.

Although it is not possible to determine exactly which antibody paratopes are present in a given serum (assuming each is possible), a limited interpretation is possible. It is clear that for all antisera tested, the diversity of the antibody paratopes represented is limited. Each serum contains antibodies having a common minimal requirement for interaction with its corresponding epitope to occur—e.g., D-L-G-S for anti-virus type A_{10} serum and D-L-G for anti-peptide type A_{10} serum. Furthermore, the fewer the absolutely required amino acids, the greater the heterogeneity of the antibody paratopes present in the serum—that is, the fewer the absolutely required amino acids, the more nearly the response of the immune system equates to that which would have been predicted from the stochastic model applied to that response (10).

By using the above reasoning it was determined that (i) of the anti-virus sera, anti-type O_1 represented a greater paratope diversity than either anti-type A_{10} or anti-type C_1 , (ii) each anti-peptide serum represented a greater paratope diversity than the comparable anti-virus serum, (iii) of the anti-peptide sera, anti- C_1 -peptide represented a much greater paratope diversity than either anti- O_1 -peptide or anti- A_{10} -peptide, and (iv) each anti-peptide serum represented a paratope diversity less than that which would have been predicted from a stochastic model for the immune response, applied to a conformationally free small peptide immunogen.

DISCUSSION

Each virus strain used in this study belongs to a different serotype. Immunity to one serotype leaves an animal susceptible to infection by virus of another serotype. Therefore, it was surprising that each of the epitopes overlaps with a group of amino acids (-G-D-L-) conserved between serotypes. The finding of conserved residues suggests that (i) residues that we were unable to identify by the method used, additional to those within the antigenic hexapeptide, may

Table 3. Relative antibody-binding activities of the single residue replacement sets of peptides with anti-peptide serum

Substituting amino acid	Parent sequences of single residue replacement sets of peptides																	
	Subtype O_1						Subtype A_{10}						Subtype C_1					
	G	D	L	Q	V	L	G	D	L	G	S	I	D	L	A	H	L	T
A	103	32			167	16				102	105	102		118	103	51	79	86
C	54	38			15	94				21	87	31		92		65	82	57
D	43	108			47	55	72	51			100	74	59	100		43	48	79
E	40	22			41	19	86				95	99	32	107		29	57	91
F	66				91	11	28					107		93		40	89	52
G	96	30			89		95			105	85	103		45	22	11	50	75
H	101	16			62		13				68	46		101		86	65	72
I	79		19		84				56		36	140		88			102	75
K	54	11			150	57	75				97	129		95		51	93	56
L	66	31	102		86	83	80		111		91	121		107	19	19	110	55
M	89	26			125	13	95				78	111		87		42	83	63
N	94	101		25	88	12	76	27	14		94	97		13		53	91	71
P	104	12					67							30				34
Q	70	26		91	137		79				99	97		119	14	62	72	79
R	77				40	12	73				91	69		78		19	63	22
S	90	38			58		60				99	102		76	30	95	99	101
T	107	12			164						30	110		66		40	85	104
V	96			10	120				23		69	124		91		12	107	62
W	59	20									42	40		60		14	76	54
Y	65			16	41	14					50	49				49	68	45

Antibody-binding activities are expressed as a percentage of the mean activity of the six parent sequences synthesized for each group. Activities are shown for all peptides for which the activity was >10%. Shown are the values obtained for anti-peptide serum when allowed to react with the homologous set of peptides. Activities shown in boldface type correspond to the values for the parent sequence. One-hundred percent values for anti-peptide sera types A_{10} , O_1 , and C_1 correspond to absorbances of 2.14, 1.34, and 0.97, respectively.

contribute to each immunogenic epitope, (ii) the conformation of the epitope of each serotype is different and that conformation contributes to the specificity of the immune response, or (iii) the specificity of the immune response is determined by only a few residues, an observation consistent with the work of others (2, 3). From the expectation that residues that contribute to the immunogenic identity of a virus would be actively selected against, it follows that these conserved amino acids may be essential for protein structure or virus function or both. Amino acid sequences of other subtypes show that the pair glycine-aspartic acid is present in all and that leucine is conserved for subtypes of type O and C but that type A viruses show variation at this position (3, 13, 14). Also seen is that the pair glycine-aspartic acid is preceded by an arginine residue in all cases except for virus type A₁₀, in which the sequence is -R-S-G-D-. It has been demonstrated recently that the tetrapeptide -R-G-D-S- constitutes the cellular recognition determinant of fibronectin and is also present in some other proteins that may interact with cells (22). It was further shown that -R-G-D- was essential for binding but that serine could be replaced by cysteine or threonine. These findings coupled with the almost absolute conservation of the grouping -R-G-D- in the VP1 protein of representative subtypes of three serotypes of FMDV raise the interesting possibility that this triplet constitutes a general recognition sequence for a cellular receptor, specificity for a given cell type being determined by the adjacent residues.

Anti-peptide sera demonstrated a greater specificity for the homologous virus than for homologous peptide. Two of the sera were able to neutralize only the homologous virus. The occurrence of isoleucine in the 6th position in the A₁₀ peptide instead of leucine, as is the case for the O₁ and C₁ peptides, may account for the low cross-reactivity of the anti-A₁₀-peptide serum for the heterologous peptides—that is, only a small number of paratopes were present in anti-A₁₀-peptide serum that could recognize the amino acid sequence of the O₁- or C₁-peptides.

Anti-peptide sera to peptides O₁ and C₁ were shown clearly to react with heterologous peptide. These same sera were less reactive with the equivalent amino acid sequence as present in the heterologous virus. This suggests that (i) conformation of the more rigid protein (compared to peptide) prohibits the binding of a proportion of the antibody paratopes present in the serum, leading to the greater observed specificity, and (ii) the necessity for antibodies to have specificity for both sequence and conformation is much greater for virus neutralization than it is for binding. This may result from the neutralizing paratopes having the highest affinity for the virus.

The above findings are well supported by the observed greater diversity of antibody paratopes present in the anti-peptide sera when compared with anti-virus sera. It is seen that although anti-peptide sera react well with the homologous protein, this is accompanied by a loss of precision in the response. It is also clear that a specificity for conformation contributes to the precision of the response. It is the origin of the conformational specificity of antibody paratopes that is at issue. Residues for which an absolute requirement is demonstrated by antibody paratopes present in anti-virus sera include a predominance of hydrophobic amino acids (leucine and alanine). These side chains would not normally be expected to be at the surface of the protein (23, 24). This is especially so in that each is adjacent to hydrophilic residues, allowing for protein folding in which the hydrophobic residues are buried internally with the hydrophilic residues at the surface. It was also surprising to find that anti-peptide sera, though showing a greater paratope diversity than anti-virus sera, contained only a limited repertoire of paratopes.

This response by the immune system to a conformationally free molecule was more limited than predicted (10). Furthermore, the degree of similarity of the antibody paratopes present in each pair of sera (anti-virus and anti-peptide) suggests a nonrandom response by the immune system.

These observations suggest a hypothesis that states that the immune system is limited in the diversity of its response to any antigen, responding in a comparable manner to a folded protein or conformationally free peptide. This may result from the mechanism of presentation of antigen during the course of immune recognition. Alternatively, the limitation may derive from a need to exceed a threshold affinity between antigen and immune system receptors, the affinity being determined largely by the residues contributing to specificity. Either way this hypothesis is consistent with the observation of the high frequency with which antibodies raised against peptides have been found to react with the native protein at the region of homology.

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